Polymerization of bacteriophage ϕ 29 replication protein p1 into protofilament sheets

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Protein p1 (85 amino acids) of the *Bacillus subtilis* phage ϕ 29 is a membrane-associated protein required for *in vivo* viral DNA replication. In the present study, we have constructed two fusion proteins, maltose-binding protein (MalE)-p1 and MalE-p1 Δ N33. By using both sedimentation assays and negative-stain electron microscopy analysis, we demonstrated that MalE-p1 molecules self-associated into long filamentous structures, which did not assemble further into larger arrays. These structures were constituted by a core of protein p1 surrounded by MalE subunits. After removal of the MalE component by cleavage with protease factor Xa, the resulting protein p1 filaments tended to associate, forming bundles. The MalE-p1 Δ N33 fusion protein, however, did not self-interact in solution. Nevertheless, after being separated from the MalE domain by factor Xa digestion, protein p1 Δ N33 assembled into long protofilaments that associated in a highly ordered, parallel array forming large two-dimensional sheets. These structures resemble eukaryotic tubulin and bacterial FtsZ polymers. In addition, we show that protein p1 influences the rate of *in vivo* ϕ 29 DNA synthesis in a temperature-dependent manner. We propose that protein p1 is a component of a viral-encoded structure that associates with the bacterial membrane. This structure would provide an anchoring site for the viral DNA replication machinery.
Keywords: membrane-associated protein/protofilament sheets/viral DNA replication

Introduction

The α/β -tubulin heterodimer is the structural subunit of microtubules, which are the cytoskeletal elements involved in cell division, mitosis and intracellular transport in all eukaryotes. Each monomer (α and β) has a relative molecular mass of 50 000 (M_r 50 kDa), and the sequences are 40–50% identical. Under some experimental conditions, purified α/β -tubulin assembles into straight protofilaments that can associate further to make two-dimensional protofilament sheets (Erickson and Stoffler, 1996; Erickson *et al.*, 1996). FtsZ (M_r 40 K) is thought to be the bacterial homolog of the eukaryotic tubulins, although the sequences are <20% identical (Erickson, 1995). Like tubulin, FtsZ has the ability to assemble into various structures including protofilament sheets and rings or spirals (Erickson *et al.*, 1996). Furthermore, FtsZ polymerization, like that of

tubulin, is dynamic and regulated by GTP hydrolysis (Yu and Margolin, 1997; Mukherjee and Lutkenhaus, 1998). However, in spite of the structural similarities, tubulin and FtsZ polymers have unrelated functions. FtsZ, which is ubiquitous in eubacteria and is also found in archaea and chloroplasts (Erickson, 1997), localizes early at the division site to form a filamentous ring-shaped septum. This protein is now recognized to play a key role in the assembly of the division apparatus and in the process of cytokinesis (reviewed by Rothfield and Justice, 1997).

Viral-encoded replication proteins able to polymerize into protofilament sheet structures have not been previously described. The genome of the *Bacillus subtilis* bacteriophage ϕ 29 consists of a linear double-stranded DNA molecule with a terminal protein (TP) covalently linked to each 5' end. The availability of an *in vitro* ϕ 29 DNA replication system has allowed the functional characterization of the early viral proteins p2 (DNA polymerase), p3 (TP), p5 (single-stranded DNA binding protein) and p6 (double-stranded DNA binding protein) (reviewed by Salas *et al.*, 1996). In addition to these replication proteins, bacteriophage ϕ 29 encodes a small (M_r 10 kDa) and early protein, p1, which is also required for *in vivo* viral DNA synthesis (Prieto *et al.*, 1989; Bravo and Salas, 1997). Nevertheless, the function of protein p1 in ϕ 29 DNA replication remains undetermined.

In vitro replication of the ϕ 29 DNA molecule starts at either DNA end, where the replication origins are located, by a protein-priming mechanism. This initiation mechanism requires the formation of a heterodimer between a free molecule of TP, which acts as a primer, and the viral DNA polymerase (reviewed by Salas *et al.*, 1996). There are some reports supporting the hypothesis that *in vivo* ϕ 29 DNA replication is a process associated with the bacterial membrane. For example, it has been shown that, near the onset of viral DNA replication, parental ϕ 29 DNA-membrane complexes are formed. The formation of these complexes requires the synthesis of early viral-encoded proteins (Ivarie and Pène, 1973). However, the viral components involved in the attachment of ϕ 29 DNA to the host membrane have not been identified. Recently, using cell fractionation techniques, we have shown that both free TP and protein p1 are membrane-associated during ϕ 29 DNA synthesis. Furthermore, when ϕ 29 DNA replication is blocked, large amounts of the viral DNA polymerase are recovered in membrane fractions, in addition to free TP and protein p1 (Bravo and Salas, 1997). Based upon these findings, we have proposed that a preliminary stage in the initiation of *in vivo* ϕ 29 DNA replication is the assembly of a multiprotein complex on the host membrane that involves at least protein p1, free TP and DNA polymerase. Membrane-association of this multiprotein complex could be mediated by both protein p1 and free TP, because these proteins have affinity

Fig. 1. Relevant features of protein p1. (A) Left end of the phage ø29 genome. The position and approximate length of the genes encoding p1, DNA polymerase (DNA pol) and terminal protein (TP) are indicated. The amino acid sequence of protein p1 is shown. Leu and Met residues of the heptad repeat units are indicated. Residues corresponding to the p1ΔN33 truncated protein are underlined. (B) The putative Shine-Dalgarno sequences (SD) for proteins p1 and p1ΔN33, and the corresponding Met start codons are shown. Translation initiation events at codon Met34 of gene 1 have been previously reported (Prieto *et al.*, 1989).

for bacterial membranes in the absence of other viral components. Once this membrane-associated complex is assembled, interaction with the ends of the parental ø29 DNA molecule (replication origins) can take place. Hence, initiation of viral DNA synthesis would proceed associated with the host membrane.

In this work we provide *in vivo* evidence on the role of protein p1 in ø29 DNA replication. We report on the construction of two fusion proteins, maltose-binding protein (malE)-p1 and MalE-p1ΔN33, the latter lacking the N-terminal 33 amino acids of protein p1. We have performed sedimentation and negative-stain electron microscopy studies of both protein preparations, digested or not with protease factor Xa. The results obtained indicate that protein p1 has the capacity to self-interact in solution forming protofilaments that can associate into two-dimensional sheets. We also show that the C-terminal 52 amino acid residues are sufficient for p1 polymerization. Furthermore, we show that protein p1 influences the rate of *in vivo* ø29 DNA synthesis in a temperature-dependent manner. Therefore, protein p1 constitutes the first example of a membrane-associated viral DNA replication protein able to assemble into protofilament sheet structures. We propose that protein p1 is a component of a viral-encoded structure that associates with the bacterial membrane. This structure would provide an anchoring site for the viral DNA replication machinery.

Results

Protein p1ΔN33 co-elutes with the MalE-p1 fusion protein

Gene 1 of the *B. subtilis* bacteriophage ø29 encodes a protein of 86 amino acids. In *Escherichia coli*, the methionine start codon is processed resulting in a protein of 85 amino acids (protein p1, Figure 1A) with a M_r of 9761 (Prieto *et al.*, 1989). To purify protein p1, gene 1 lacking the methionine start codon was inserted into the pMAL-c2 vector downstream from the *malE* gene, which encodes

Fig. 2. Co-elution of MalE-p1 and p1ΔN33. The MalE-p1 fusion protein was purified by binding to an amylose column. The fusion protein was eluted from the column with 10 mM maltose. Column fractions of 1 ml were collected, and aliquots from such fractions were analyzed by SDS-PAGE (15% polyacrylamide). The amount of both MalE-p1 and p1ΔN33 in each fraction (arbitrary units) was determined by densitometric scanning of the gel stained with Coomassie Blue.

MalE. The resulting MalE-p1 fusion protein (52 kDa) was purified by affinity to amylose columns and eluted with maltose. Unexpectedly, a protein of ~6 kDa co-eluted with the MalE-p1 fusion protein (Figure 2). This small protein was identified as p1ΔN33 by determination of its N-terminal sequence. Synthesis of p1ΔN33 is probably due

to a translation initiation event at codon Met34 of gene 1 (Figure 1B). The molar ratio of MalE-p1 to p1ΔN33 was ~5. The co-elution suggested that protein p1ΔN33 could interact with the p1 domain of the MalE-p1 chimeric protein. Therefore, a region of protein p1 located between residues Met34 and Lys85 may be involved in p1-p1 interactions. This result was the first evidence that protein p1 may self-interact. According to computer algorithms (Lupas *et al.*, 1991), the region of protein p1 located between Leu37 and Asn66 presumably forms an α -helical coiled-coil structure (heptad repeat region) (Figure 1A). This structural element has been extensively involved in protein-protein interactions (Lupas, 1996).

Polymerization of the MalE-p1 fusion protein

Preliminary experiments (not shown) indicated that ~40% of the eluted MalE-p1 hybrid protein (concentrated to 14 μ M) co-sedimented with protein p1ΔN33 during ultracentrifugation. This observation suggested that a fraction of the MalE-p1 molecules were aggregated, and protein p1ΔN33 was a component of such aggregates.

To characterize the aggregated form of the MalE-p1 fusion protein, we analyzed the eluted MalE-p1 protein preparation by sedimentation through glycerol gradients. As shown in Figure 3A, two populations of the chimeric protein were separated (fractions I and II). Approximately 30% of the MalE-p1 fusion protein sedimented at the position of 85 kDa (fraction I), calculated by loading different marker proteins in the same gradient (Figure 3B). The small protein p1ΔN33 was not detected in this population (Figure 3A). Thus, it is likely that fraction I corresponds to the monomeric form of the MalE-p1 hybrid protein. The other MalE-p1 population (fraction II) showed a rapid velocity of sedimentation and it contained the p1ΔN33 truncated protein (Figure 3A). When fraction II was analyzed by negative-stain electron microscopy, filamentous structures of various lengths and ~18 nm in diameter were observed (Figure 4A). These filaments were not visualized after treatment of the MalE-p1 protein preparation with proteinase K (not shown). Interestingly, ~20% of the MalE-p1 protein recovered from fraction II (protein filaments) sedimented at the monomeric position after dialysis and loading into a second glycerol gradient (disassembly). Moreover, MalE-p1 fusion protein sedimenting at the monomeric position (fraction I) was able to reassemble after dialysis and concentration to 7 μ M. At this concentration, MalE-p1 filaments identical to those formed in the presence of protein p1ΔN33 (Figure 4A) were visualized by negative staining.

From these results we conclude that MalE-p1 chimeric molecules are able to self-associate as a function of protein concentration. This self-association leads to the formation of filamentous structures. MalE-p1 assembly does not require protein p1ΔN33, but molecules of p1ΔN33 can interact with MalE-p1 molecules and become components of the protein filaments. In addition, these results indicated that protein p1 was acting as a polymerization domain, since protein MalE does not self-interact in solution (see below).

Assembly of protein p1 into filaments

To investigate the ability of protein p1 to polymerize in solution, we performed sedimentation assays and electron

Fig. 3. Sedimentation studies of the MalE-p1 protein preparation eluted from the amylose column. (A) Two hundred microliters of a MalE-p1 protein preparation (27 μ M) were loaded onto a 15–30% glycerol gradient, and subjected to centrifugation for 9 h as indicated in Materials and methods. After fractionation of the gradient, aliquots from each fraction were analyzed by SDS-PAGE (15% polyacrylamide). Densitometric scanning of the gel stained with Coomassie Blue was used to determine the amount of MalE-p1 (arbitrary units) in each fraction. Fractions 1–8 (population II) and fractions 18–25 (population I) were pooled, dialyzed against dialysis buffer (see Materials and methods) and concentrated to 8 μ M. Samples from both populations were analyzed by SDS-Tricine-PAGE (Schagger and Jagow, 1987). As a control, an aliquot of the MalE-p1 protein preparation loaded onto the glycerol gradient was analyzed in the same gel (T). (B) Fifty microliters of the MalE-p1 protein preparation (27 μ M) were subjected to centrifugation in a 15–30% glycerol gradient for 21 h as indicated in Materials and methods. As markers, β -galactosidase (116 kDa), bovine serum albumin (BSA; 67 kDa) and ϕ 29 terminal protein (TP; 31 kDa) were loaded in the same gradient.

microscopy analysis of MalE-p1 protein preparations digested with protease factor Xa. This protease cleaves after the sequence IEGR, which separates the MalE and p1 domains. For this study, a MalE-p1 protein preparation purified from p1ΔN33 (Fraction I, Figure 3) was concentrated to 7 μ M, conditions in which MalE-p1 molecules assemble into filaments.

A totally digested MalE-p1 protein preparation was ultracentrifuged (Figure 5A). Under our ultracentrifugation conditions, protein p1 sedimented whereas the MalE

Fig. 4. MalE-p1 filaments visualized by electron microscopy. (A) Fractions 1–8 (population II) from the glycerol gradient shown in Figure 3A were pooled and dialyzed. Then, the preparation was treated for negative staining. (B) MalE-p1 filaments were partially digested with protease factor Xa as indicated in Materials and methods. A sample of such digestion was diluted and observed by negative staining.

domain remained in the supernatant. Analysis of this digested MalE-p1 protein preparation by sedimentation through glycerol gradients demonstrated that MalE behaved as a monomer after being separated from protein p1 (Figure 5B), while protein p1 was essentially found at the bottom of the gradient (not shown). Hence, these sedimentation assays showed that protein p1, but not MalE, was able to form large structures in solution. This conclusion was confirmed by negative-stain electron microscopy analysis of MalE-p1 protein preparations partially or totally digested with protease factor Xa. As shown in Figure 4B, partial digestion of MalE-p1 polymers revealed that these structures were constituted by a filamentous core with a diameter of ~6 nm. Furthermore, protein filaments with this diameter and different lengths were visualized in totally digested MalE-p1 protein preparations (Figure 6A), although these thin filaments tended to associate into bundles (Figure 6B).

Taking the above results together, we conclude that self-association of MalE-p1 molecules is exclusively mediated by p1–p1 interactions. The assembly process leads to the formation of filamentous structures constituted by a core of protein p1. In these polymers, the MalE domain of every MalE-p1 molecule may be oriented toward the surface. After removal of the MalE subunits by protease factor Xa, the resulting p1 filaments tend to associate into bundles, suggesting that the presence of the MalE domain limits the formation of new, larger arrangements. Thus, we conclude that protein p1 has the capacity to polymerize in solution.

Protein p1 Δ N33 assembles into protofilament sheets

Co-elution of the small protein p1 Δ N33 with the MalE-p1 fusion protein (Figure 2), as well as its co-sedimentation with MalE-p1 polymers (Figure 3A), suggested that the N-terminal 33 amino acids of protein p1 were dispensable for p1–p1 interactions.

To find out whether the amino acid residues between Met34 and Lys85 (Figure 1A) are sufficient for p1 polymerization, we constructed the MalE-p1 Δ N33 fusion protein. This purified protein was not able to self-interact in solution, as determined by sedimentation assays through glycerol gradients. In such experiments, only one population of MalE-p1 Δ N33 molecules sedimenting at the monomeric position was observed (not shown). This behavior of the MalE-p1 Δ N33 fusion protein was crucial to study the assembly properties of protein p1 Δ N33 in solution. For this analysis, we carried out kinetic experiments in which the fate of the p1 Δ N33 molecules after addition of factor Xa to a MalE-p1 Δ N33 preparation was followed by both ultracentrifugation assays (Figure 7) and negative-stain electron microscopy (Figure 8). After 9 h of factor Xa addition, the concentration of p1 Δ N33 was 11 μ M. At this time, protein p1 Δ N33 did not sediment during ultracentrifugation, and negatively-stained specimens were not visualized. However, at 12 h, when the concentration of p1 Δ N33 was 13 μ M, ~60% of the p1 Δ N33 protein molecules sedimented, whereas MalE was recovered in the supernatant. Thus, formation of large p1 Δ N33 structures was taking place. Analysis of this preparation by

Fig. 5. Sedimentation studies of MalE-p1 protein preparations totally digested with protease factor Xa. **(A)** A MalE-p1 protein preparation from fraction I (see sedimentation through the glycerol gradient shown in Figure 3) was dialyzed and concentrated to 7 μ M. Under these conditions, MalE-p1 filaments were formed. This preparation was totally digested with factor Xa. The digested preparation was ultracentrifuged as indicated in Materials and methods. In this experiment, the sedimented material was concentrated 2-fold in loading buffer. Equivalent volumes of the supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE (15% polyacrylamide). An aliquot of the digested MalE-p1 preparation taken before ultracentrifugation (T) was run in the same gel. **(B)** The totally digested MalE-p1 protein preparation was subjected to ultracentrifugation in a 15–30% glycerol gradient for 20 h as indicated in Materials and methods. Under these conditions, protein p1 was recovered at the bottom. Bovine serum albumin (BSA) (67 kDa) and ϕ 29 TP (31 kDa) were loaded in the same gradient as markers.

electron microscopy showed that long protofilaments associated in a highly ordered, parallel array were formed. These structures were two-dimensional sheets. The protofilament sheets had different lengths, and their width was usually in the range of 14–62 nm. Electron micrographs of these negatively-stained structures taken at 15 h, when the concentration of p1 Δ N33 was 14 μ M, are shown in Figure 8. Between 15 and 21 h after adding factor Xa, the concentration of p1 Δ N33 did not significantly change (Figure 7). Nevertheless, the percentage of p1 Δ N33 molecules sedimented during ultracentrifugation increased slightly over time (from 73 to 91%), indicating that they become components of large structures. Furthermore, as

p1 Δ N33 assembly proceeded, the length of the protofilament sheets increased, and sheets of up to 85 nm in width were occasionally observed.

We conclude that the C-terminal 52 amino acid residues of protein p1 are sufficient to generate protofilament sheet structures. In addition, we conclude that steric hindrance mediated by the MalE domain prevents self-interaction of the MalE-p1 Δ N33 molecules.

Protein p1 ensures an efficient ϕ 29 DNA replication in vivo

Protein p1-encoding gene is located at the left end of the ϕ 29 genome, adjacent to the DNA polymerase-encoding gene (Figure 1A). Initiation of *in vitro* ϕ 29 DNA replication requires the formation of a heterodimer between a free molecule of TP, which acts as a primer, and the viral DNA polymerase. This heterodimer interacts with the origins of replication, located at both ends of the viral genome (reviewed by Salas *et al.*, 1996). During *in vivo* viral DNA replication, large amounts of the early proteins p1 and free TP are found associated with the bacterial membrane, as well as a small percentage of the total viral DNA polymerase. This fraction presumably corresponds to DNA polymerase molecules that are interacting with TP, involved in the initiation and transition stages of viral DNA replication (Méndez *et al.*, 1997). In fact, the number of DNA polymerase molecules relative to that of protein p1 and free TP increased in membrane fractions when viral DNA replication was blocked (Bravo and Salas, 1997). Based on these studies, we postulated that protein p1 is a component of a replication complex which assembles on the host membrane prior to initiation of viral DNA replication.

To understand the role of the membrane-associated p1 protein in ϕ 29 DNA replication, we measured the rate of viral DNA synthesis in the absence of protein p1 under different bacterial growth conditions. For this study, we used the ϕ 29 *sus1*(629) mutant phage (Reilly *et al.*, 1973), which has a point mutation in the protein p1-encoding gene. This mutation results in the replacement of a CAA codon (Gln at position 6, Figure 1A) by a nonsense TAA codon (Prieto *et al.*, 1989). Thus, protein p1 is not synthesized in ϕ 29 *sus1*(629)-infected non-suppressor (*su*⁻) cells. When *su*⁻ cells grown in LB medium at 30°C were infected with the ϕ 29 *sus1*(629) phage, the rate of viral DNA synthesis was slightly slower than that measured in cells infected with the wild-type phage (Figure 9A). Accordingly, the amount of ϕ 29 *sus1*(629)-DNA accumulated for 60 min was slightly less than that accumulated in wild-type ϕ 29 infected cells. However, when *su*⁻ cells grown in LB medium at 37°C were infected with the ϕ 29 *sus1*(629) phage, viral DNA synthesis was markedly delayed, and the amount of phage DNA accumulated for 40 min was ~3.5-fold lower than that accumulated in wild-type ϕ 29 infected cells (Figure 9B). Therefore, in the absence of protein p1, the rate of viral DNA synthesis decreases, and the extent of this reduction depends on the bacterial growth temperature. The drastic reduction in the rate of ϕ 29 *sus1*(629)-DNA synthesis observed in LB medium at 37°C is not related to the bacterial growth rate (20 min at 37°C compared with 45 min at 30°C), because no synthesis of ϕ 29 *sus1*(629)-DNA was detected when bacteria were grown in a defined medium at 37°C,

Fig. 6. Electron micrographs of protein p1 structures. A MalE-p1 protein preparation (7 μ M) totally digested with factor Xa was treated for negative staining. (A) The upper panel shows p1 filaments not associated into bundles. These images were occasionally observed. Note that small regions of the p1 filament shown at the bottom remain undigested. (B) After removing the MalE component, the resulting p1 filaments tended to bundle, forming large aggregates. These structures were predominant in the preparation.

Fig. 7. Polymerization of the p1 Δ N33 truncated protein. A MalE-p1 Δ N33 protein preparation, concentrated to 17 μ M, was treated with protease factor Xa. Time zero corresponds to the preparation before protease addition. Factor Xa cleavage was carried out at a ratio of 1.25% (w/w) the amount of fusion protein. The reaction mixture was incubated at room temperature. At the indicated times, samples (100 μ l) were taken and ultracentrifuged as indicated in Materials and methods. Equivalent amounts of the total (T, before ultracentrifugation), supernatant (S) and pellet (P) fractions were analyzed by SDS-Tricine-PAGE (Schagger and Jagow, 1987). The upper part of the gel was stained with Coomassie Blue. The lower part of the gel was stained with silver (Morrissey, 1981). To determine the concentration of p1 Δ N33 in the reaction mixture, the percentage of undigested MalE-p1 Δ N33 was calculated by densitometric scanning of the gel.

conditions in which the bacterial growth rate was 42 min (Bravo and Salas, 1997). Finally, to rule out the possibility that the drastic reduction in the rate of *sus1*(629)-DNA synthesis at 37°C was due to an additional temperature-sensitive mutation in the ϕ 29 *sus1*(629) mutant phage, suppressor (*su*⁺) cells grown in LB medium at 37°C were infected with the wild-type or *sus1*(629) mutant phages. As

shown in Figure 9C, both phages showed a similar rate of DNA synthesis.

From these results we conclude that protein p1 stimulates *in vivo* the rate of viral DNA synthesis. In addition, we conclude that protein p1 plays a critical role in *in vivo* viral DNA synthesis when bacteria are growing at high, rather than low, temperatures.

Fig. 8. Protofilament sheets formed by protein p1 Δ N33. Protease factor Xa was added to a Male-p1 Δ N33 protein preparation (see legend to Figure 7). At different times after protease addition, samples were diluted and treated for negative staining. Only electron micrographs taken after 15 h of factor Xa addition are shown. (A) The upper panel shows quite long protofilament sheets crossing each other. As shown at the bottom, sheets with different widths (18, 39 and 68 nm, respectively) were visualized. Note that the sheets appear folded. (B) Negatively stained protofilament sheets photographed at higher magnification. These sheets are 46 and 75 nm wide, respectively.

Fig. 9. Phage DNA replication in the absence of protein p1. Time course of the accumulation of viral DNA under one-step phage growth conditions. *Bacillus subtilis* cells were infected with either wild-type (○) or *sus1*(629) mutant (●) phages. Phage addition marked the zero time of the experiments. At the times (min) indicated on the top of the gels, total intracellular DNA was isolated and analyzed by agarose-gel electrophoresis. Proteinase K-treated ϕ 29 DNA (50 ng) was run in the same gel (lane C, internal marker). (A) *Bacillus subtilis* 110NA (*su*⁻) cells were grown in LB medium at 30°C. (B) *Bacillus subtilis* 110NA (*su*⁻) cells were grown in LB medium at 37°C. (C) *Bacillus subtilis* MO-99 (*su*⁺³) were grown in LB medium at 37°C.

Discussion

Assembly of protein p1 into protofilament sheets

Phage ϕ 29-encoded p1 protein, involved in ϕ 29 DNA replication, polymerizes in solution. A remarkable finding is that the C-terminal 52 amino acid residues of protein p1 are sufficient for assembly into protofilament sheets. Hence, this is the first report on a viral DNA replication protein which is able to generate such structures.

Purified MalE-p1 fusion protein assembles into long filamentous structures, which are constituted by a core of protein p1 surrounded by MalE subunits. Only p1-p1 interactions are involved in the formation of these polymers. The MalE-p1 filaments do not assemble further into larger arrays, most likely due to steric hindrance determined by the MalE subunits. In fact, when they are removed by cleavage with protease factor Xa, the resulting p1 filaments tend to associate forming bundles. Therefore, the presence of the MalE domain prevents interactions between p1 filaments.

The monomeric state of the MalE-p1 Δ N33 fusion protein has been crucial to obtain additional information on the assembly properties of protein p1 in solution. Protein p1 Δ N33, after being separated from the MalE domain, assembles into large polymers that show a parallel array of longitudinal protofilaments. These structures resemble protofilament sheets formed by purified α/β -tubulin, which are the main components of eukaryotic microtubules, and by FtsZ, which forms the cytoskeletal framework for cell division in all bacteria (Gaskin and Kress, 1977; Simon and Salmon, 1990; Erickson *et al.*, 1996). However, tubulin and FtsZ polymerization, but not p1 Δ N33 assembly, requires GTP (Yu and Margolin, 1997; Mukherjee and Lutkenhaus, 1998).

Although the intact microtubule is a helical polymer, its assembly involves primarily the formation of small protofilament sheets. These two-dimensional polymers grow longer by adding subunits at the end, and wider by initiating new protofilaments (Erickson, 1974; Voter and Erickson, 1984; Simon and Salmon, 1990). Studies on microtubule nucleation support the notion that assembly of a protein into protofilament sheets requires a very precise sequence of events. We believe that such conditions are provided by the MalE-p1 Δ N33 fusion protein because it does not self-interact in solution. Consequently, after adding protease factor Xa to a MalE-p1 Δ N33 preparation, p1 Δ N33 molecules begin to accumulate and, when a critical concentration is reached, they assemble into protofilament sheets without any interference from the MalE-p1 Δ N33 molecules still present in the preparation. A different situation occurs in MalE-p1 protein preparations. In this case, the MalE-p1 molecules are able to self-interact, but the presence of the MalE domain imposes a restriction to the growth of the polymer, allowing exclusively the formation of long filaments.

Assembly of protein p1 Δ N33 into protofilament sheets takes place in the absence of auxiliary proteins or factors, suggesting that the information necessary to form such polymers relies upon its structure. According to computer algorithms (Lupas *et al.*, 1991), the region of protein p1 located between Leu37 and Asn66 probably forms an α -helical coiled-coil structure. Therefore, this short α -helical segment may be involved in the intermolecular interactions

required for polymerization. In fact, coiled-coils are the main structural elements in the assembly of intermediate filament proteins (reviewed in Herrmann and Aebi, 1998). Intermediate filaments have been recognized as a superfamily of 10 nm fibers ubiquitous in multicellular eukaryotes. Despite the rather low primary sequence identity, all the intermediate filament proteins share a common molecular architecture. They have a central α -helical domain, which is flanked by non-helical end domains. In contrast to protein p1, the α -helical domain is very long (~42 heptads). This domain has the ability to form two-stranded parallel coiled coils. In addition, dimer-dimer interactions are implicated in the intermediate filament assembly.

In vivo role of protein p1

In vivo ϕ 29 DNA replication is a process associated with the bacterial membrane (Ivarie and Pène, 1973; Bravo and Salas, 1997). We have previously shown that protein p1 behaves as a membrane-associated protein in infected cells, both during synthesis of ϕ 29 DNA and after blocking ϕ 29 DNA replication. Moreover, membrane-association of protein p1 also occurs in the absence of other viral components, indicating that protein p1 has affinity for bacterial membranes (Bravo and Salas, 1997). Protein p1 influences the rate of *in vivo* viral DNA synthesis in a temperature-dependent manner. Although the absence of protein p1 slightly reduces the rate of ϕ 29 DNA synthesis at low temperatures (30°C), protein p1 plays a critical role on *in vivo* viral DNA replication when bacteria are growing at high temperatures (37°C). The requirement of protein p1 for an efficient viral DNA replication at high, rather than low, temperatures could be related to temperature-dependent modifications in the composition of membrane phospholipids. *Bacillus subtilis* cells, in common with other bacteria, synthesize phospholipids with a greater proportion of saturated fatty acids when grown at high, rather than low, temperatures. These modifications are thought to be designed to attenuate the effects of temperature changes on the fluidity of the cell membrane (de Mendoza and Cronan, 1983). This adaptation seems to be particularly important for soil bacteria like *B. subtilis*. Therefore, protein p1 appears to ensure an efficient viral DNA replication under particular bacterial growth conditions.

It is still unknown whether p1 polymerizes *in vivo* and, if this is the case, what kind of structures p1 would form. It has been argued that the geometry of a protein molecule that can assemble into a protofilament sheet is so complex and precise that this assembly could not exist *in vitro* unless it were constantly selected for, and hence, functionally important *in vivo* (Erickson *et al.*, 1996). Furthermore, we have reported that at early stages of ϕ 29 infection there are $\sim 10^4$ copies of protein p1 per infected cell, and it increases up to 10^5 at late stages (Bravo and Salas, 1997). Thus, taking into account the cell volume of infected cells (Abril *et al.*, 1997), the intracellular concentration of protein p1 would range from 16 to 160 μ M throughout the infective cycle, which is sufficient to obtain polymerization *in vitro*. These considerations suggest that protein p1 could be able to polymerize *in vivo*, being that such a state is essential for its function. We speculate that the functional p1 polymer *in vivo* could be based on the protofilament

sheet structure formed by protein p1 Δ N33. Some questions remain open such as the length and width of the putative p1 protofilament sheets formed *in vivo*, and whether other viral and/or host proteins might influence p1 polymerization under physiological conditions.

The ability of protein p1 to bind to membranes *in vivo*, and to polymerize *in vitro*, suggests that protein p1 could assemble *in vivo* to form a viral structure in close association with the bacterial membrane. Alternatively, this membrane-associated viral structure might be an assembly of different proteins, including protein p1. Thus, protein p1 would contribute to stabilize the attachment of such viral structure to membranes under certain bacterial growth conditions. The nuclear lamina, a thin fibrous structure immediately underlying the inner nuclear membrane of most eukaryotic cell nuclei, is an assembly of up to five different lamin proteins. Lamins constitute a group of intermediate filament proteins (Herrmann and Aebi, 1998). Among other functions, the nuclear lamina may be involved in the organization of chromatin and, consequently, may influence DNA replication and transcription during interphase (Moir *et al.*, 1995). The putative membrane-associated ϕ 29-encoded structure might provide a site for recruitment of viral replication proteins prior to initiation of ϕ 29 DNA synthesis. In fact, when *in vivo* ϕ 29 DNA replication is blocked, large amounts of viral DNA polymerase are sequestered in membranes, together with protein p1 and free TP (primer) (Bravo and Salas, 1997).

In conclusion, our results indicate that protein p1 has the capacity to assemble *in vitro* forming protofilament sheet structures, and that this ability is located within the C-terminal 52 amino acid residues of the protein. *In vivo*, protein p1, which has affinity for membranes, ensures an efficient ϕ 29 DNA replication under particular bacterial growth conditions. We propose that protein p1 is a component of a membrane-associated viral-encoded structure. This structure would provide a foundation for assembly of the viral DNA replication machinery, leading to the compartmentalization of the viral DNA replication process.

Materials and methods

Bacterial strains, plasmids, bacteriophages and growth conditions

Escherichia coli XL1-Blue (purchased from Stratagene) was used as host for the plasmids constructed in this work. To construct plasmid pMalE-p1, ϕ 29 DNA sequences from coordinate 1130–809 (Yoshikawa and Ito, 1982) were amplified by polymerase chain reaction (PCR) using the oligonucleotides *Sma*I (5'-GGAGATGTTTGTACCCGGGAAAAT-CTTCG-3') and *Xba*I (5'-CCGTCATCATCTAGAAGCAAC-3') as primers. To construct plasmid pMalE-p1 Δ N33, ϕ 29 DNA sequences from coordinate 1036–809 were amplified by PCR using the oligonucleotides *Sma*I Δ N33 (5'-CCTGTAGACGGAGACCCCGGGATGATAGACC-3') and *Xba*I (see above). In both constructions, PCR fragments were digested with *Sma*I and *Xba*I, and ligated into the *Xmn*I and *Xba*I sites of plasmid pMAL-c2 (New England Biolabs). The nucleotide sequence of the inserts was confirmed by DNA sequencing using the dideoxy nucleotide chain-termination method (Sanger *et al.*, 1977) with Sequenase (United States Biochemicals sequencing kit). Therefore, plasmid pMalE-p1 carries gene 1 of ϕ 29 lacking the methionine start codon downstream from the Ile-Glu-Gly-Arg encoding sequence. In the case of plasmid pMalE-p1 Δ N33, there is a Gly codon between the Ile-Glu-Gly-Arg encoding sequence and the Met34 codon of gene 1. Protease factor Xa cleaves after the sequence Ile-Glu-Gly-Arg. Plasmid-containing cells were grown in LB-broth (Sambrook *et al.*, 1989) supplemented with ampicillin (100 μ g/ml).

Bacillus subtilis 110NA (Moreno *et al.*, 1974) was used as non-suppressor (*su*⁻) strain. *Bacillus subtilis* MO-99 (Moreno *et al.*, 1974) was used as suppressor strain (*su*⁺). The ϕ 29 *sus*1(629) mutant phage was isolated by Reilly *et al.* (1973). Phage stocks were prepared essentially as reported (Talavera *et al.*, 1971), except that the phage particles were concentrated as described (Bravo *et al.*, 1990). LB medium was supplemented with 5 mM MgSO₄ in phage infection experiments.

Purification of the MalE fusion proteins

Escherichia coli cells carrying a recombinant plasmid based on the pMAL-c2 vector were exponentially grown in selective medium at 34°C. When the OD₅₆₀ was 0.45, production of the fusion protein was induced by adding isopropyl-1-thio- β -D-galactopyranoside (IPTG) to a final concentration of 2 mM. The culture was then incubated for 2 h at the same temperature. After this induction period, cells were concentrated 10-fold in column buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 10 mM β -mercaptoethanol), and disrupted by French pressure treatment (20 000 p.s.i.). The cell extract was centrifuged at 10 000 r.p.m. for 45 min at 4°C in a Sorvall SS.34 rotor. The supernatant was collected and diluted 2.5-fold in column buffer. This cleared lysate was loaded onto an amylose-agarose column (New England Biolabs), previously equilibrated with column buffer. The resin was washed extensively with the same buffer before bound proteins were eluted with elution buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM β -mercaptoethanol, 10 mM maltose). Aliquots from each fraction were run in a SDS-polyacrylamide gel, and fractions containing the fusion protein were pooled. Protein preparations were concentrated using a Microcon microconcentrator 10 (Amicon).

Factor Xa protease cleavage

Protein preparations were dialyzed against 20 mM Tris-HCl pH 7.5, 200 mM NaCl and 10 mM β -mercaptoethanol (also referred to as dialysis buffer). If necessary, protein preparations were concentrated to at least 0.3 mg/ml, and 1 μ g of factor Xa (New England Biolabs) was added to 25–100 μ g of the fusion protein. Usually, the reaction mixture was incubated at 4°C. The cleavage with factor Xa was monitored by SDS-PAGE.

Sedimentation assays

Ultracentrifugation of protein preparations (100–200 μ l) was carried out at 40 000 r.p.m. for 1 h at 4°C in a Beckman TLA.45 rotor. After centrifugation, the supernatant was collected, and the sedimented material was usually resuspended in the same volume of loading buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 5% β -mercaptoethanol and 30% glycerol).

Sedimentation through glycerol gradients was performed as follows. The indicated amount of protein was loaded onto a 5 ml glycerol gradient (15–30% in column buffer) prepared in a Beckman polyallomer centrifuge tube (13 $\times$ 51 mm). Centrifugation was performed at 62 000 r.p.m. and 4°C in a Beckman SW.65 rotor for the indicated time. After centrifugation, 170 μ l fractions were collected by puncturing the bottom of the tubes. Aliquots from each fraction were analyzed by SDS-PAGE.

Electron microscopy

Protein preparations were diluted to 0.5–1.5 μ M in dialysis buffer (see above), and immediately applied to carbon-coated copper grids for 2 min. Grids were then washed with a few drops of water and stained for 30 s with 1% uranyl acetate. Electron micrographs were taken in a Jeol 1010 electron microscope at 80 kV.

Synthesis of viral DNA

The method used to measure synthesis of viral DNA under one-step phage growth conditions was as described (Bravo *et al.*, 1994). Basically, *B. subtilis* cells were exponentially grown to $\sim 10^8$ cells/ml, and then infected with the indicated phage at a multiplicity of infection of 5. After 10 min incubation with gentle shaking, unadsorbed phages were eliminated by centrifugation of the infected culture. Cells were resuspended in the same volume of pre-warmed medium and incubated with vigorous shaking at the same temperature. Total intracellular DNA was isolated at different times after infection, and analyzed by 0.8% agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed under ultraviolet light illumination. Bands corresponding to unit-length ϕ 29 DNA were quantified by densitometry of the negatives in a Molecular Dynamics 300A densitometer. The relative amount of viral DNA refers to the intensity of the intracellular ϕ 29 DNA band with respect to that of proteinase K-treated ϕ 29 DNA (50 ng) used as internal marker. In these experiments, the high multiplicity of infection, together with the removal of unadsorbed phage particles, ensures that

the whole cell population is simultaneously infected. Consequently, the infection process takes place synchronously and kinetic analyses can be carried out.

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